FINAL REPORT

Remediation of Perfluoroalkyl Contaminated Aquifers
Using an In-situ Two-layer Barrier:
Laboratory Batch and Column Study

SERDP Project ER-2127

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1. Introduction

1.1 Background

This ER-2127 project is a Limited Scope SEED project with a fixed budget of $150K, starting in June 2011 and scheduled to complete in May 2014 after no cost extension. The project is proof of concept in nature, which was designed to investigate the feasibility of a novel scheme for the remediation of groundwater contaminated by perfluoroalkyl chemicals (PFCs). This approach is derived from our previous finding that PFCs can be effectively transformed during enzyme-catalyzed humification reactions in the presence of phenolic substrates as mediators (Colosi et al. 2009). The phenolic mediators generate highly reactive radical intermediates under the enzyme catalysis which in turn initiate fortuitous secondary reactions of PFCs, leading to their degradation. The primary objective of this study is to verify the feasibility of using a permeable reactive barrier system to induce effective enzyme-catalyzed humification reactions for in-situ remediation of groundwater PFC contamination.

PFCs, such as perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), have extremely high thermal and chemical stability, stemming from their unique structural features in which all hydrogens on carbon are replaced with fluorines. Carbon-fluorine bond is strong because fluorine has strong electronegativity that induces a partial positive charge on carbon and a negative charge on fluorine atoms, leading to electrostatic attraction that makes the bond shorter and stronger. Fluorine is the most electronegative element having a reduction potential of 3.6 V (Wardman 1989). It is thus thermodynamically unfavorable to oxidatively replace the fluorine atom with any other atom. In addition, the fluorine atoms, which are much larger than hydrogen in size, form a dense hydrophobic layer in PFOX (X=S or X=A) surrounding carbon-carbon bonds, which shields them from attack by oxidative reagents. The oxidation resistance prevents PFCs from being oxidized under normal conditions or utilized by microbes as carbon and energy sources, making these chemicals extremely persistent in the environment.

PFOA and PFOS in groundwater can be removed by *ex situ* liquid phase granular activated carbon; however, the high stability of PFCs renders current *in situ* treatment technologies involving oxidation and microbial degradation not so effective for degrading PFCs (Vecitis et al. 2009). Advanced oxidation processes (AOPs), such as alkaline ozonation, peroxone, or Fenton's reagent, have been shown effective to degrade a wide range of organic contaminants. The degradation largely relies on the oxidative power of hydroxyl radicals generated during these processes. Hydroxyl radicals normally react with saturated organics through an H-atom abstraction, or react with unsaturated organics via an oxidative addition reaction. However, PFCs have no hydrogen available for abstraction, or a double bond for addition, leaving direct electron transfer the only possible pathway which is unfortunately less potent. Thus, the perfluorination in PFC renders these compounds essentially inert to those advanced oxidation techniques (Vecitis et al. 2009). A couple of direct and indirect photolytic oxidation pathways (Hori et al. 2005, Kormann et al. 1991, Kutsuna and Hori 2007), sonochemistry (Cheng et al. 2008, 2010), and reductive dehalogenation have been shown effective for PFC degradation, but are not applicable for *in situ* treatment applications.

We made an important finding in an earlier study that PFOA can be effectively degraded during enzyme-catalyzed oxidative humification reactions (ECOHR) (Colosi et al. 2009), which sheds light on an alternative approach to PFC treatment and remediation. ECOHR refers to a class of oxidative reactions critically involved in natural organic matter (NOM) humification processes. These reactions are ubiquitous in soil systems, and usually catalyzed by certain naturally occurring extracellular enzymes that are produced by certain white/brown rot fungi, including lignin peroxidase, manganese peroxidase and laccases, etc. (Bollag 1992, Dec and Bollag 2000). These enzymes effectively catalyze conversion of natural or anthropogenic chemicals containing phenolic or anilinic moieties into active intermediates such as radicals or quinones that can subsequently be bound covalently into NOM (Colosi et al. 2009, Huang and Weber 2004a, b, Park et al. 1999, Weber and Huang 2003). Such active intermediates may also fortuitously attack other inert chemicals, such as lignin, and thus cause their degradation and consequently incorporate them into the natural humification process. We have shown in our earlier studies that such mediator-facilitated mechanism can lead to degradation of PFCs (Colosi et al. 2009), PAHs (Weber and Huang 2003), and PCBs (Colosi et al. 2007) during ECOHR. A number of naturally occurring chemicals, such as 4-methoxyphenol, guaiacol, catechol, and even soil organic matter itself can serve as such mediators by generating free radicals under humification enzyme catalysis (Colosi et al. 2007, Colosi et al. 2009, Weber and Huang 2003). There are two classes of enzymes that are able to catalyze ECHOHR, including certain peroxidases, such as lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and horseradish peroxidase (HRP, EC 1.11.1.7), and some phenol oxidases, such as laccase (E.C. 1.10.3.2). Peroxidases catalyze the oxidation of phenolic chemicals into free radicals in the presence of hydrogen peroxide, while phenol oxidases carry out phenol oxidation in the presence of oxygen.

In our previous study (Colosi et al. 2009), we observed PFOA degradation during ECOHR mediated by horseradish peroxidase (HRP) in the presence of 4-methoxyphenol as a co-substrate under room temperature and at a neutral pH. Such reactions did not occur in the absence of the 4-methoxyphenol, suggesting that HRP's interaction with 4-methoxyphenol as a mediator resulted in PFOA degradation. Based on the products pattern, we postulated that the phenoxyl radicals formed during HRP oxidation of 4-methoxyphenol fortuitously attacked PFOA, likely initiated by abstracting an electron from the head C-C bond on PFOA, leading to its breakdown. It is not fully clear yet why phenoxyl radicals appear to be more effective in transforming PFOA than hydroxyl radicals generated in AOPs, but it may be related to the longer life time of phenoxyl radicals and its relative hydrophobicity. The PFC molecules contain a dense hydrophobic outer layer of fluorine atoms that shield C-C bonds. The hydrophobic phenoxyl radicals may be more amenable than hydrophilic hydroxyl radicals to approach the C-C bonds and initiate PFC breakdown.

1.2 Objective

The finding described above suggests that PFCs may be transformed in the natural environment through humification, although such natural process may be very slow given the low concentration of PFCs and the low dosages of humification enzymes in the natural environment. However, it is possible that such humification reactions of PFCs can be enhanced through engineering approach in treatment or remediation applications. **We hypothesize that a permeable reactive barrier system that induces effective humification reactions by**

supplementing appropriate enzymes and cofactors can be used in the remediation of groundwater flows containing PFCs.

We originally conceived a double-layer permeable reactive barrier (DL-PRB) system, shown in Figure 1, for inducing *in situ* ECHOR. The DL-PRB comprises an oxidant-releasing material layer followed by a layer of quartz sands immobilized with humification enzymes. The oxidant-releasing material layer, which contains calcium peroxide, continuously releases oxidants (oxygen and hydrogen peroxide) upon contact with water, which feeds into the humification enzyme layer where humification processes are facilitated to result in concomitant transformation of PFCs. The primary objective of this study was to verify the feasibility of using this conceptual DL-PRB system for *in-situ* remediation of groundwater PFC contamination.

Figure 1 Scheme of two-layer barrier in column study
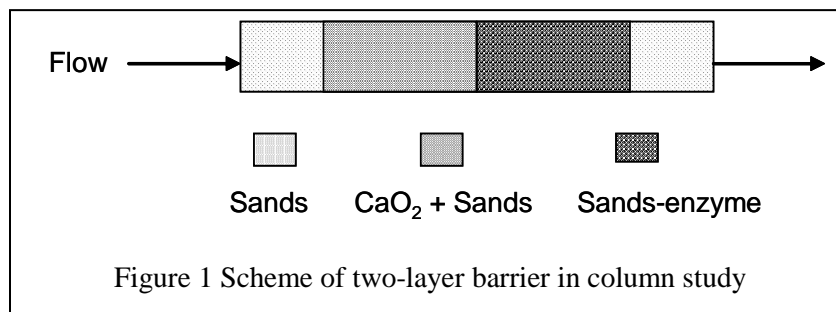
We proposed to conduct four research tasks in order to achieve the above mentioned objective. Task I comprised batch reactor studies aimed to optimize ECHOR conditions for enhanced PFC degradation. Task II focused on enzyme immobilization for use in the PRB setup. Task III was intended to study the use of calcium peroxide as a source of oxidants for ECHOR in anaerobic condition. Task IV was a laboratory column study to evaluate the performance of the PRB system to remove PFCs in a water flow.

1.3 Report Organization

We have made significant progress in Tasks I, II and IV studies and the results are presented below. In Task I we have investigated PFC degradation in ECHOR in two types of systems: aqueous phase or soil slurry, and their results are presented in Sections 2 and 3, respectively. The results from Task II study on immobilization of humification enzymes on sand and other media are presented in Section 4. The results of a column study in Task IV are described in Section 5. Each section contains an experiment, a result and discussion, and a conclusion and future research need subsection.

2. Batch Reactor Study: Aqueous Phase

We have conducted a series of experiments to examine PFOA degradation during ECHOR in aqueous phase under various combinations of different conditions, including three different enzymes (HRP, LiP and Laccase) at different dosages, five different mediators (guaiacol, catechol, HBT, veratryl alcohol, and 4-methoxyphenol) at different concentrations, and different initial PFOA concentrations. The goal was to verify PFOA degradation in different ECHOR systems and explore the range of effective conditions.

2.1 Experiments

The experiments examining ECHOR in aqueous phase were carried out at room temperature in test tubes, each containing 5 mL solution having PFOA, a mediator, and a humification enzyme, all at systematically varied concentrations. When peroxidases were used, hydrogen peroxide was also included in the solution and was added last to start the reaction. Each condition was tested with triplicate. Systems without enzyme or a mediator added were also tested for comparison. The test tubes were caped and incubated for pre-determined times with agitation. At the end of incubation, 0.5 mL of the reaction solution was withdrawn from the reactor and mixed with 0.5 mL methanol to terminate the reaction, and the mixture was used for LC-MS/MS analysis to quantify PFOA.

Quantification of PFOA was performed on a Waters Micromass Quattra tandem mass spectrometer (Waters, Milford, MA) interfaced with a Waters 2690 HPLC system (Waters, Milford, MA). The separation was performed on an ascentic C18 reversed phase column (25 X 4 mm, 5µm, Supelco, St. Louis, MO). A binary gradient elution consisting of 2 mM ammonium acetate solution in deionized water (A) and acetonitrile (B) at a flow rate of 0.3 mL/min was used as mobile phase for PFOA quantification. The injection volume was 10 µL. The gradient spanned 30 minutes and was programmed as follows: 40% B was increased linearly to 80% at 5 minutes, 90% at 10 minutes, held at 90% B for additional 15 min and finally decreased to 40% B. The electrospray ionization in a negative mode was used for both PFOA and PFOS detection with the following parameters: capillary voltage was set to 3.01 V, desolvation temperature to 300 ^{o}C, and source block temperature to 100 ^{o}C. For PFOA, cone voltage set to 20 V and collision energy at 20 eV, while for PFOS cone voltage and collision energy were set at 67 V and 38 eV respectively. Nitrogen (Airgas, > 99.999% purity) was used as nebulizer and drying gas and gas flow rates were maintained at 34, and 198 L/hour, respectively. PFOA were quantified by multiple reaction monitoring (MRM) using the most abundant precursor/ product (m/z) ion transition (413→369 for PFOA). External five point calibration curves for this method were generated using standard PFOA concentrations ranging from 0.01 mg/L to 10 mg/L.

2.2 Results and Discussion

The experimental results from aqueous phase reactions are summarized in Table 1. PFOA removal percentage was calculated by comparing the reaction sample with the control sample which was prepared the same as the reaction sample except for the absence of enzyme. A couple observations can be made. First, all three tested enzymes can cause PFOA removal under certain conditions, as seen in the systems 13 and 14 for HRP, 16-18 for LiP, and 22-54 for laccase. Second, when the initial PFOA concentration was above 100 µM, no significant PFOA concentration reduction was observed, as shown in the systems 1-12 and 55-57. Third, the best PFOA removal percentage was nearly 30% (systems 27, 41, 53 etc.), and increase of enzyme dosage (e.g., systems 23, 35, 41) or mediator concentration (e.g., systems 22-24) did not significantly enhance PFOA removal. This suggests that a factor other than enzyme dosage or mediator concentration may have limited the extent of PFOA removal in the tested reaction systems.

Table 1. PFOA removal under various ECHOR conditions in aqueous phase

No.	PFOA Con. (μM)	Enzyme Type	Mediator Type	E dosage (U/mL)	M dosage (μM)	Reaction Time (h)	Removal (%)	standard deviation
1			Guaiacol	0.015	10	1	0.02	0.43
2					100		0.30	1.08
3					1,000		2.00	0.16
4					10,000		-0.35	0.34
5			Catechol	0.015	10	1	2.10	0.59
6	100	HRP[1]			100		6.10	0.58
7					1,000		1.10	0.13
8					10,000		6.98	4.07
9			HBT	0.015	10	1	3.40	0.28
10					100		3.50	0.41
11					1,000		2.00	0.04
12					10,000		5.40	6.39
13	24	HRP[1]	4-methoxy-phenol	0.02	210	2	12.10	2.55
14				0.1	210		19.73	7.82
15				0.005	0		3.70	-
16	30	LiP[2]	Veratryl alcohol		200	1	23.10	-
17				0.025	0		18.20	-
18					200		19.20	-
19					20		0.97	4.03
20					200	2	3.49	3.73
21					2,000		1.41	2.10
22					20		19.53	7.00
23					200	24	21.56	10.14
24				0.3	2,000		17.48	1.84
25					20		26.20	5.30
26					200	72	27.20	1.46
27					2,000		27.10	7.97
28					20		21.64	1.05
29					200	120[4]	20.46	10.23
30					2,000		21.44	0.72
31					20		11.07	5.68
32					200	2	12.45	1.82
33					2,000		6.13	4.63
34					20		10.87	0.00
35					200	24	8.62	3.49
36	1	Laccase[3]	4-methoxy-phenol	1	2,000		12.48	12.62
37					20		23.60	4.80
38					200	72	16.40	19.02
39					2,000		11.30	8.13
40					20		27.06	3.89
41					200	120[4]	21.53	2.76
42					2,000		19.82	0.28
43					20		9.86	6.21
44					200	2	10.80	13.29
45					2,000		5.90	0.59
46					20		22.69	4.13
47					200	24	20.00	5.21
48				10	2,000		19.60	0.67
49					20		6.00	5.89
50					200	72	20.80	0.36
51					2,000		9.40	2.21
52					20		27.32	0.96
53					200	120[4]	30.43	0.67
54					2,000		17.54	0.09
55					20		5.17	1.17
56	1,000	Laccase[3]	4-methoxy-phenol	10	200	24	-4.60	10.09
57					2,000		-14.67	3.74

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1: H_2O_2 concentration is 150 µM for all horseradish peroxidase (HRP) reaction. 2: H_2O_2 concentration is 100 µM for all lignin peroxidase (LiP) reaction 3: the O_2 required for laccase reaction was naturally dissolved oxygen. 4: The mediator was added at the designated dosage once at time 0 and once at 72h.

Because all three tested enzymes exhibited the capability of mediating ECHOR to cause PFOA degradation, we identified laccase as the most promising candidate for potential remediation use based on our knowledge of the humification enzymes and our experiment results from other related research. First, laccase is highly stable, retaining high enzyme activity during ECHOR. All humification enzymes undergo inactivation during ECHOR via certain suicidal mechanisms (Colosi et al. 2006), but our experiments to be discussed below in Figures 2 and 3 revealed that laccase inactivation was much slower. Laccase still retained significant activity after reactions for 3 days in aqueous phase and 45 days in soil slurry, while our earlier studies have shown that peroxidases lost their activities quickly after several hours of ECHOR in aqueous phase (Huang et al. 2002, Mao et al. 2009). Second, laccase has wide industrial application, e.g. textile and paper and pulp, and are thus readily commercially available in large quantity and reasonable price. Third, laccase uses oxygen as cofactor while peroxidases use hydrogen peroxide. As such, use of laccase in potential remediation practice would avoid the need of hydrogen peroxide addition. Therefore, our further experiments have focused on laccase.

In order to explore factors controlling PFOA degradation during laccase-mediated ECHOR, we further characterized selected reaction systems with regard to enzyme activity change and mediator degradation, and the results are shown in Figure 2. As seen in Figure 2, the laccase activity remained relatively stable during the 3 day reaction in all systems. The mediator 4-methoxyphenol diminished quickly during the first 2 hours of reaction, indicating the occurrence of ECHOR. It is known that laccase mediates the oxidative reaction of 4-methoxyphenol to form 4-methoxyphenyl free radical during ECHOR. The free radicals are highly reactive, which will couple to form dimer and polymers (Mao et al. 2009) or react with PFOA to cause its degradation (Colosi et al. 2009). These dimers and polymers are still substrates of laccase that can undergo further ECHOR that may have contributed to the ensuing degradation of PFOA after the first few hours of reaction. Figure 2B shows that 4-methoxyphenol concentration was reduced to 19.4% after 2 hours of reaction and further to 11.3% after 72 hours of reaction in the presence of 0.3 units/mL laccase. When laccase activity was increased to 10 units/mL, as shown in Figure 2D, the reduction of 4-methoxyphenol concentration was 17.2% and 11.8% after 2 and 72 hours of reaction, respectively, not much different from the systems with 0.3 units/mL laccase. This suggests that laccase was used in excess with regard to mediator in these systems, and this may be why no significant enhancement of PFOA removal was achieved by increasing enzyme dosages in these systems.

By comparing Figures 2A, 2B, and 2C, it may be seen that as the mediator concentration increased from 20 to 2000 µM in the presence of 0.3 units/mL laccase PFOA removal was not enhanced much, although the absolute quantity of 4-methoxyphenol transformed during ECHOR increased nearly 100 fold. This suggests that there may be another unidentified factor that has limited the extent of PFOA degradation during ECHOR. Laccase consumes oxygen as cofactor during ECHOR, and in our experiment systems this was naturally dissolved oxygen without extra supply and the reactor was closed. Although the continued 4-methoxyphenol degradation in our experiment systems suggests the presence of oxygen but it would have decreased over the

course of ECHOR and thus limited the reaction rate. In addition, oxygen may not only serve as laccase cofactor in ECHOR systems but also directly participate in PFOA degradation. It has been suggested 4-mehoxyphenol converts into 4-mehoxyphenyl free radicals upon ECHOR and these free radicals attack PFOA, likely on the head C-C bond to abstract an electron, and turn PFOA into a free radical that can undergo further rearrangement and degradation (Colosi et al. 2009). An earlier study on electrochemical degradation of PFOA has indicated that PFOA was turned into a free radical electrochemically which then degraded through rearrangements, and oxygen was involved in this process and incorporated in the products (Zhuo et al. 2011). As such, consumption of oxygen during ECHOR may not only limit laccase activity but also limit the rearrangement and degradation process of PFOA free radicals.
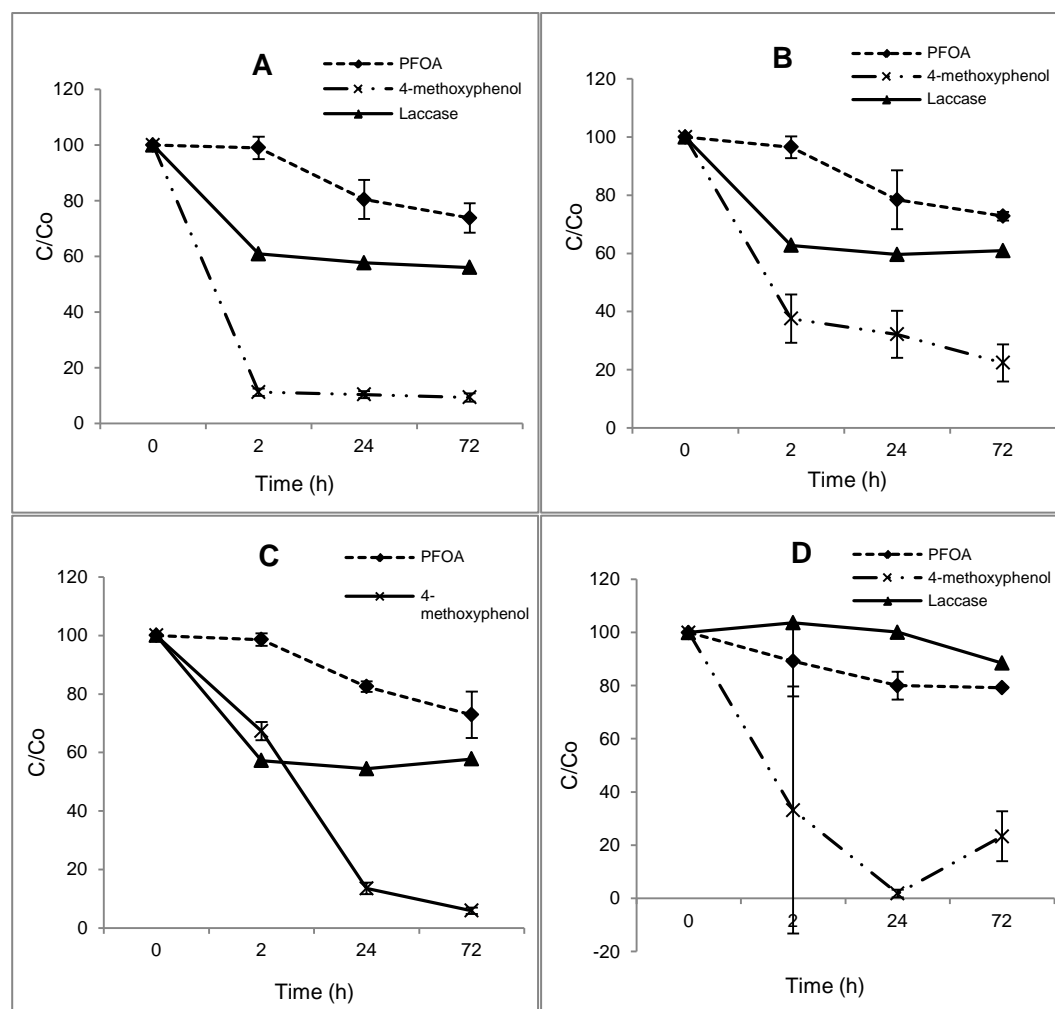
Figure 2. Normalized PFOA and 4-methoxyphenol concentrations and laccase activity as a function of time in aqueous phase. The starting concentrations are 0.83 µM PFOA for all treatments, the laccase activity and 4-methoxyphenol concentration are **A**: 0.3 unit/mL laccase and 20 µM 4-methoxyphenol; **B**: 0.3 unit/mL laccase and 200 µM 4-methoxyphenol; **C**: 0.3 unit/mL laccase and 2,000 µM 4-methoxyphenol; and **D**: 10 unit/mL laccase and 200 µM 4-methoxyphenol.

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2.3 Conclusion and research need

Our experimental results indicate that all three enzymes are capable of mediating ECHOR to cause PFOA degradation under appropriate conditions. Laccase is the most promising candidate among all three enzymes for potential remediation use because of its stability and availability. The level of oxygen may be a critical factor that influences PFOA degradation during ECHOR in addition to laccase dosage and mediator concentration, which needs to be further verified in future study. The use of isotope-labeled oxygen in combination with product identification may help to explore the role of oxygen in PFOA degradation. Further study on product identification is needed also because it will help understand PFOA degradation mechanisms during ECHOR and possible product toxicity. Combination of mass spectrometry and chemical oxidation technology as well as high resolution mass spectrometry and nuclear magnetic resonance may be useful in product identification.

3. Batch Reactor Study: Soil Slurry

We have also conducted experiments to examine both PFOA and PFOS degradation during laccase-mediated ECHOR in soil slurry. These experiments were done because we have found in a recent study for a different project that the reaction behavior of ECHOR is very different between in aqueous phase and in soil slurry. This is not surprising because soil interacts with the enzyme, PFOA, and the mediator, which would thus influence the interactions among the three factors and in turn change ECHOR dynamics. Thus, the influence of the presence of soil on PFC degradation during ECHOR needs to be understood for effective use of ECHOR in groundwater remediation. We in this study employed similar experiment conditions that have been identified in a different study that have caused effective degradation of hormones in soil slurry systems.

3.1 Experiment

Incubation. Incubation experiments were conducted with sterilized soil containing PFOA or PFOS in the presence of laccase and different mediators. Before each incubation experiment, the soil was sterilized by incubation twice, 24 hours each time, and autoclaved three times, 60 min each time, at 121°C over a three day period. To prepare PFOA or PFOS contaminated soil samples, 100 g soil was spiked with PFOA or PFOS stock solution in methanol, which was then left uncovered under fume hood with vigorous mixing to evaporate solvent and yield a sample containing 10 µg PFOA g^{-1} soil or 5 µg PFOS g^{-1} soil. The property of the soil used in this study is given in Table 2.

Table 2. Property of the tested soil

Parameter	Value	Parameter	Value
Sand content (%)	66.00	Cation exchange capacity (meq/100 g)	3.70
Silt content (%)	23.60	Organic matter content (%)	2.90
Clay content (%)	10.40	Soluble salts (mmhos/cm)	0.04
pH	5.15	Base saturation (%)	65.93

The incubation was conducted in 50 mL polypropylene centrifuge tubes. Each tube contained 1 g soil (preloaded with 10 µg PFOA or 5 µg PFOS) and 1.5 mL distilled deionized water. Laccase was added to the system at 2 unit g^{-1} soil every 48 hrs. One of the three mediators violuric acid (VA), 1-hydroxybenzotriazole (HBT), and 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was added along with laccase each time. The amount of mediator added each time was equal and the total amount added was either 4 or 8 µmole over the entire incubation period. Control systems were also prepared without laccase or mediator. All tubes were placed in dark throughout the experiment, with openings covered with paraffin film to allow exchange of oxygen while restrict water evaporation. The contents of each tube was thoroughly mixed on a mechanical shaker at the beginning of the incubation and then mixed manually twice a day during the entire incubation period. The paraffin film was left open for hour after each mixing to allow sufficient re-aeration. At pre-selected time intervals, triplicate tubes were sampled for PFOA and PFOS analysis. To this end, the samples were first frozen at -18 ^{o}C and then freeze dried (Labconco freeze drier) for further extraction of PFOA and PFOS from soil as described below.

Extraction of PFOA from soil: The soil in each reactor, after being freeze dried as mentioned above, was mixed with a 5 mL mixture of dichloromethane and methanol (2:1, V:V), ultrasonicated for 30 minutes, and then centrifuged for 30 minutes at 250 g. This extraction procedure was repeated once. Supernatant from both extractions was combined and then dried to 1 mL under a gentle nitrogen flow. The solution was then loaded onto a silica solid-phase extraction (SPE) cartridge (Restek, 6 mL, 1000 mg) that was preconditioned with 5 mL of hexane. After loading, the cartridge was eluted with 20 mL hexane and air dried for 5 min for cleanup. The cartridge was then eluted with 5 mL acetonitrile, with the eluent collected, blown dry by a nitrogen flow, and finally reconstituted to 1.0 mL with acetonitrile for PFOA or PFOS quantification on LC-MS/MS using the same method described in 2.1.

3.2 Results and discussion

Figure 3 displays the recovery of applied PFOA (%) from the laccase-treated soil samples after 15 or 45 days of incubation. PFOA recovery in the control system (without laccase or mediator added) was near 85% and consistent, reflecting the robustness of our analytic procedure. Note that PFOA recovery in laccase-treated samples (without added mediator) was 70.2 and 65.6% after 15 and 45 days of incubation, respectively, much lower than the control systems. This indicates that PFOA was transformed during laccase-mediated ECOHR even without a mediator added. It is known that PFOA is not a direct substrate of laccase, and its transformation occurs via a mediator-assisted mechanism (Colosi et al. 2009). Such mediator, usually a phenolic chemical, reacts with humification enzymes to generate a free radical that can in turn attack PFOA. The fact that PFOA was transformed in laccase-mediated soil slurry without an added mediator may suggest that the soil organic matter in soil (or dissolved from soil) can serve the role of mediator and induce PFOA transformation during ECHOR. This finding is of important implication in the potential use of ECHOR in PFC remediation. There has been report that soil organic matter contains rich phenolic functionalities and can react with humification enzymes (Piccolo et al. 2000).

Figure 3 also shows that PFOA recovery in laccase-treated soil was significantly reduced when a mediator was added. This suggests the importance of mediator and corroborates the notion that PFC is transformed in ECHOR via a mediator-assisted mechanism. For laccase-VA, laccase-HBT, and laccase-ABTS systems, the values of PFOA recovery was 11.6, 27.7, and 19.9% for 15 day incubation, and 9.9, 1.6, and 5.3% for 45 day treatment, respectively. The PFOA removal extents were significantly greater than those obtained in aqueous phase experiments as shown in Table 1 and Figure 1. Although the reaction conditions were not directly comparable between the aqueous phase and soil slurry experiments, the presence of soil may have played a role to facilitate ECHOR and PFOA degradation. One phenomenon that we have observed is that enzyme inactivation was mitigated in soil slurry. In a test we have observed that near 50% laccase activity remained after 45 days of ECHOR in soil slurry. It is possible that the laccase may be sorbed on soil and thus be protected from inactivation, and there have been reports that sorption of humification enzymes on support media effectively mitigate enzyme inactivation (Eichlerová et al. 2012). Further, the soil may also effectively sorb PFOA and the mediators in addition to the enzyme, and thus all ECHOR factors were concentrated on soil surface, which may have also facilitated ECHOR through so-called micro-reactor effect.

Figure 4 compares PFOA recovery after 15 day incubation with each of the three mediators added at two different dosages, 4 or 8 micromole g^{-1} soil. At the lower mediator dosage (4 micromole g^{-1} soil), PFOA recovery in laccase-VA, laccase-HBT, and laccase-ABTS systems was 34.8, 42.1 and 41.8% respectively, while at 8 micromoles g^{-1} soil, the values were 11.6, 27.7, and 19.9%. The dependence of PFOA removal on mediator dosage is evident with higher mediator dosage corresponding to lower PFOA recovery. This seems to differ from what we observed for aqueous phase shown in Figure 1 where increase of mediator concentration from 20 to 2000 µM had little impact on PFOA removal. Note that in the soil slurry experiment paraffin film was used to cover the reaction vessels and it was left open periodically to allow sufficient re-aeration, while the reactors in aqueous phase experiment was caped through the experiment. It might be that the limited oxygen supply has limited PFOA removal in the aqueous phase experiment, and when this limitation factor was alleviated in the soil slurry experiment the effect of mediator concentrations became evident.

Figure 5 displays the recovery of applied PFOS (%) from the laccase-treated soil samples after 15 days of incubation. PFOS recovery in the control system (without laccase or mediator added) was 81.4 %, while that in laccase-treated samples (without added mediator) was reduced to 65.8%. When a mediator was added, PFOS recovery was further reduced to 17.8, 9.1 and 10.0 % in laccase-VA, laccase-HBT, and laccase-ABTS systems. This is a strong indication of PFOS transformation in ECHOR. All earlier reports on PFC degradation in ECHOR was focused on PFOA and our data here is the first set showing PFOS degradation, which is significant and encouraging. It is also important to note that PFOS, like PFOA, also degrades in laccase-treated systems without an added mediator. This suggests that soil organic matter can serve the role of mediator in ECHOR to induce the degradation of both PFOA and PFOS. It is of practical significance to further examine this and explore if PFC degradation may be accelerated by adding natural organic matter.
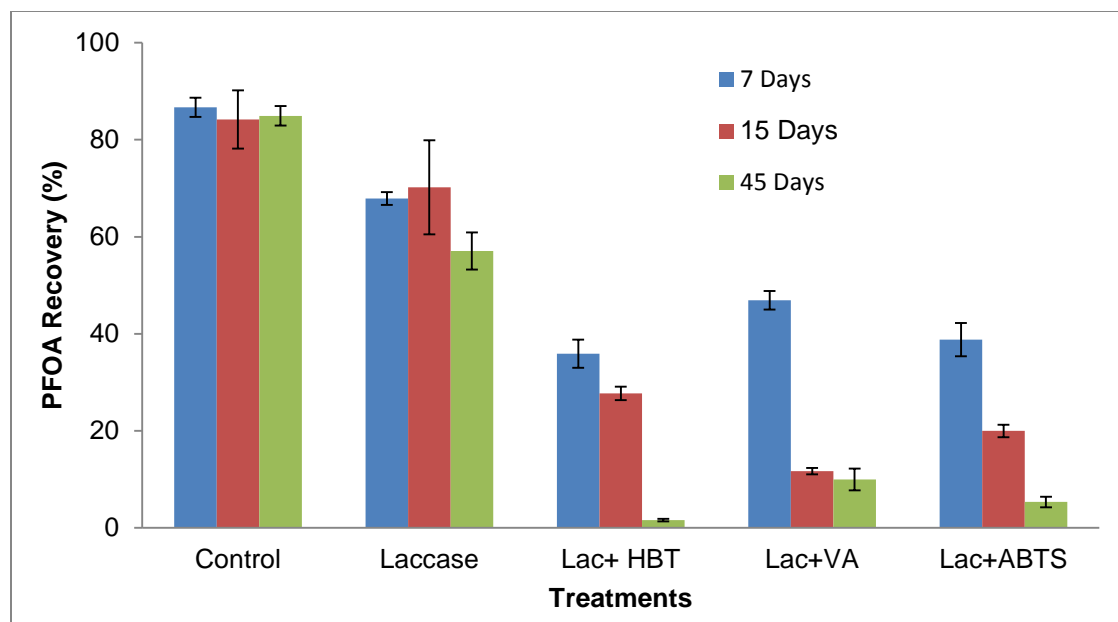
Figure 3. PFOA recovery (%) after 7, 15 and 45 days of incubation. Each reactor contained 1g soil preloaded with 10 µg PFOA in 1.5 mL water. Laccase was added every 48 hours at 2 units/g soil, and the mediator (HBT, VA or ABTS) was added along with laccase. The amount of mediator added each time was equal and the total amount added was 8 µmoles/g soil. Values are the means of three replicates and error bars represent standard deviations.
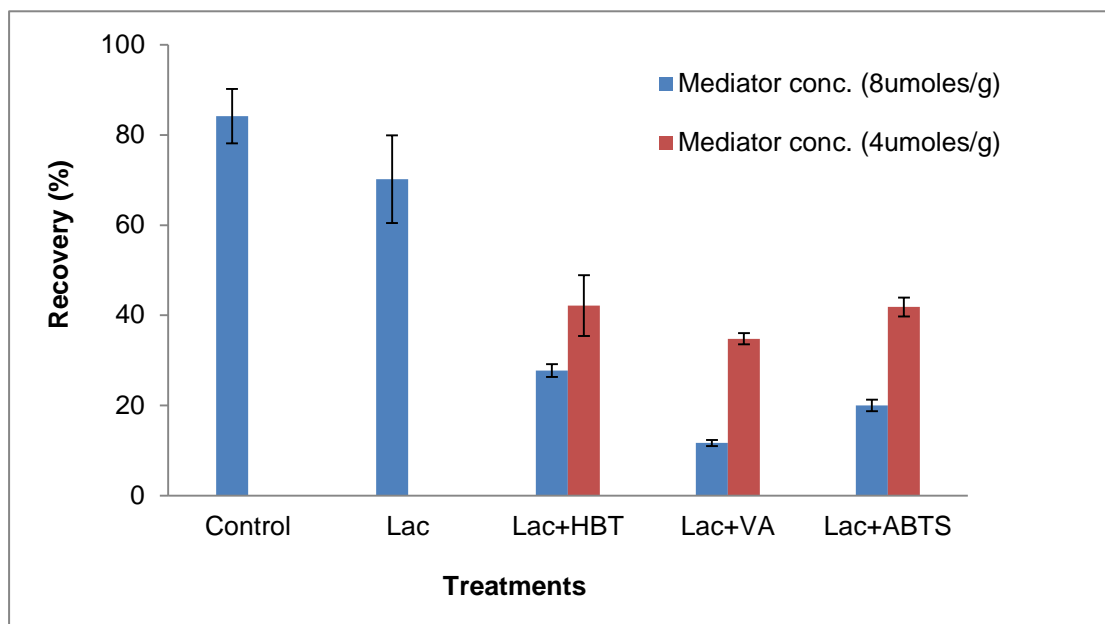
Figure 4. PFOA recovery (%) after 15 days of treatment. Each reactor contained 1g soil preloaded with 10 µg PFOA in 1.5 mL water. Laccase was added every 48 hours at 2 units/g soil, and the mediator (HBT, VA or ABTS) was added along with laccase. The amount of mediator added each time was equal and the total amount added was 4 or 8 µmole/g soil. Values are the means of three replicates and error bars represent standard deviations.

Figure 5. PFOS recovery (%) after 15 days of incubation. Each reactor contained 1g soil preloaded with 5 µg PFOS in 1.5 mL water. Laccase was added every 48 hours at 2 units/g soil, and the mediator (HBT, VA or ABTS) was added along with laccase. The amount of mediator added each time was equal and the total amount added was 8 µmole/g soil. Values are the means of three replicates and error bars represent standard deviations.
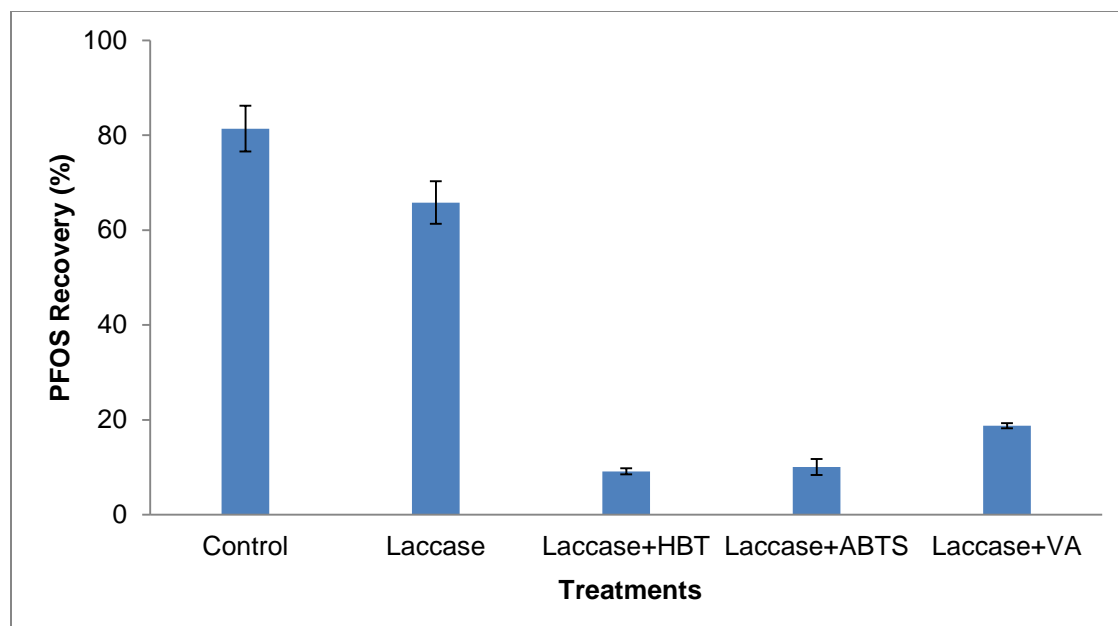
3.3 Conclusion and future research needs

Our experiments have shown that both PFOA and PFOS were degraded significantly in soil via laccase-mediated ECHOR. It seems that soil organic matter can serve the role of mediator for ECHOR to cause PFC degradation, while the reaction was enhanced when chemical mediators were added. It appears that the presence of soil in the system plays a synergetic role in ECHOR, which warrants further investigation in future study. Soil may sorb laccase and protect it from inactivation, thus leading to prolonged catalytic activity. All ECHOR factors, including the mediator, the enzyme and PFOA, may be concentrated on soil surface via sorption which may enhance the reaction by micro-reactor effect. Further understanding of these effects and more detailed knowledge of the interactions among ECHOR factors and different soil components will enable science-informed design and optimization of ECHOR in soil remediation.

4. Enzyme Immobilization

We have studied several approaches, including layer-by-layer assembly, covalent bonding and adsorption, to immobilize two humification enzymes, horseradish peroxidase (HRP) and laccase, onto the surface of various support media. Quartz sand, soil, clay and activated carbon were used as the support media. Optimum immobilization conditions for different enzymes and immobilization methods have been identified. The goal was to develop approaches

for preparing enzyme-loaded solid medium that can be used in the PRB to induce ECHOR for PFC remediation.

4.1 Experiments

4.1.1 Enzyme immobilization

Layer-by-layer assembly approach

The layer-by-layer assembly approach for enzyme immobilization is based on the strategy of alternately depositing layers of oppositely charged polyelectrolytes or enzymes on the support medium surface (Caruso and Schüler 2000, Huang et al. 2008). These layers were held strongly together through electrostatic interactions. We have tested this approach on sand as a model support medium and the procedure is briefly described below.

Prior to the assembly of polyelectrolyte multilayers, sands were cleaned up by RCA procedure with 5:1:1 (vol) $H_2O/H_2O_2/HCl$ mixture at 70^oC for 10 min followed by 10 min of immersion in 5:1:1 (vol) $H_2O/H_2O_2/NH_4OH$ solution at 70^oC. The assembly involved alternate deposition of polyelectrolytes, or the charged enzyme on treated sand. Each sorption step was carried out in a separate solution with pH deliberately adjusted to render the polyelectrolytes or the enzyme having appropriate charges. Each sorption step led to a reversal of the terminal surface charge after adsorption of a new layer. Following a conventional layer-by-layer method, we used poly(allylamine hydrochloride) (PAH) and poly(sodium 4-styrenesulfonate) (PSS) as the polyanions and polycations, respectively (Caruso and Schüler 2000, Huang et al. 2008). Both chemicals were prepared at a concentration of 100 mg/mL in 0.05 M sodium acetate buffer at pH 4.5. The pH of the enzyme solution was adjusted to several units away from their isoelectric points ($pI_{laccase} = 3.7$, $pI_{HRP} = 8.8$) to maintain a net negative or positive charge. Sequential polyelectrolyte/enzyme layers were deposited to form repetitive sand-PAH-PSS-enzyme or sand-PAH-PSS-PAH-enzyme sandwich assemblies. For each assembly step, different concentrations of polyelectrolyte/enzyme solution were allowed to equilibrate with the sand particles for 20 min and 2 hours for polyelectrolyte and enzyme, respectively, at different temperatures.

Covalent bonding approach

The covalent binding approach for enzyme immobilization involves first activation to attach reactive functional groups on the support medium surface followed by covalent bonding to tether the enzymes under appropriate reaction conditions (Fernández-Fernández et al. , Sarkar et al. 1989, Yang et al. 2010). We have tested this approach on sand, soil and clay as model support media, and the procedures are described briefly below.

Sand. 10 g sands were mixed with 50 mL of 2% chitosan solution with stirring for 1 hour and then rinsed with deionized (DI) water several times. The chitosan coated sands were then treated with 10% of gultaraldehyde with continuously stirring for 2 hours. The sands were collected and rinsed with DI water and dried in the oven. 1g pretreated sands were mixed with 5 mL of an enzyme solution at different dosages and pH that contained 0.5% gultaraldehyde and stirred for 2 hours under various temperatures.

Soil and clay. The soil with property displayed in Table 2 and the clay kaolinite were used in this study. 1g soil or clay was treated with concentrated HNO_3 with boiling for 1 hour. Then the pellet was rinsed with DI water several times till the pH of rinse water reached 6.0. To activate the support medium, the pretreated soil or clay was immersed in 2% solution of 3-aminopropyltriethoxysilane (APTES) in acetone. The activated support medium was then treated with 5 mL of 5% glutaraldehyde dissolved in 100 mM phosphate buffer (pH 7.0). The mixture was then evacuated for 1 h and washed several times with DI water and then with 100 mM pH 7.0 phosphate buffer. The enzyme was prepared in different pH buffers to make 100 unit/mL solutions, which was mixed with the activated support medium, and the mixture was incubated at 4^oC for 36 hours with occasional shaking for enzyme immobilization.

Adsorption approach

Certain solid support media can strongly adsorb enzymes via non-specific physical interactions, which would also lead to effective enzyme immobilization. We have tested this approach on granular activated carbon (GAC).

Batch adsorption experiments were carried out in 125 mL conical flasks by mixing 50 mg of granular activated carbon (20-40 mesh) with 10 mL of an enzyme solution at different dosage. The mixture was incubated on an incubator shaker operated at a constant agitation speed of 125 rpm under 25^oC for 72 hours, which was sufficiently long to attain equilibrium as verified in preliminary tests. After incubation, the solution phase and solid phase was separated by centrifugation. The laccase activity remaining in solution before and after the adsorption was quantified by spectrometry described below.

4.1.2 Enzyme activity assessment

HRP activity was assayed by measuring the color change of ABTS. In this method 0.05 mL of an enzyme solution sample was added to a 3 mL of reaction mixture containing 2 mM ABTS in phosphate buffer (pH 6.0). This was followed by addition of 0.3 mL of 10 mM H_2O_2 to the mixture and the absorbance was measured at 405 nm on a Du 640-B spectrophotometer (Beckman Instruments, Inc.). One unit of peroxidase activity is defined as the amount catalyzing the oxidation of one µmol of ABTS per minute. Laccase activity was determined spectrometrically by oxidation of 1 mM 2, 6-dimethoxyphenol in citrate phosphate buffer (pH 3.8), the absorbance of which was measured at 468 nm. One unit of laccase activity is defined as the amount of enzyme that causes a unit change per minute in absorbance at 468 nm in 3.4 mL of this solution in cuvette with 1 cm light path. When assessing the activity of enzyme immobilized on a support medium, the enzyme-loaded solid sample was collected, washed thoroughly with DI water till no further activity present in the rinse. 10 mg of the solid sample was used for activity assessment using the same methods described above except that the assay solution was shaken during the incubation and centrifuged before absorbance measurement.

4.2 Results and discussion

Layer-by-layer approach

Figure 6 presents the results of HRP immobilization when different concentration of HRP solution was used in the layer-by-layer assembly procedure, while Figure 7 shows those when the HRP solution was adjusted to different pH. Generally speaking, the activity of immobilized

HRP increases as more enzyme layers were assembled on the support surface. As seen in Figure 6, higher HRP activity was immobilized when higher concentration of the enzyme solution was used in assembly. Shown in Figure 7, immobilized HRP activity increased when the immobilization pH rose from 4.5 to 6, but it dropped sharply when the immobilization pH was around 7.5 (Figure 7). This was because it was closer to the pI_{HRP} (pH 8.8) and thus HRP became not fully charged. The result of laccase immobilization is shown in Figure 8. The immobilized laccase activity also increased with the increase number of enzyme layers. The elevation of immobilized laccase activity with the decrease of pH was observed. However, the immobilization efficiency for laccase was quite low, only at 0.054 unit/ g sand under the optimum condition. This indicated that layer-by-layer approach is not an option of choice for laccase immobilization.
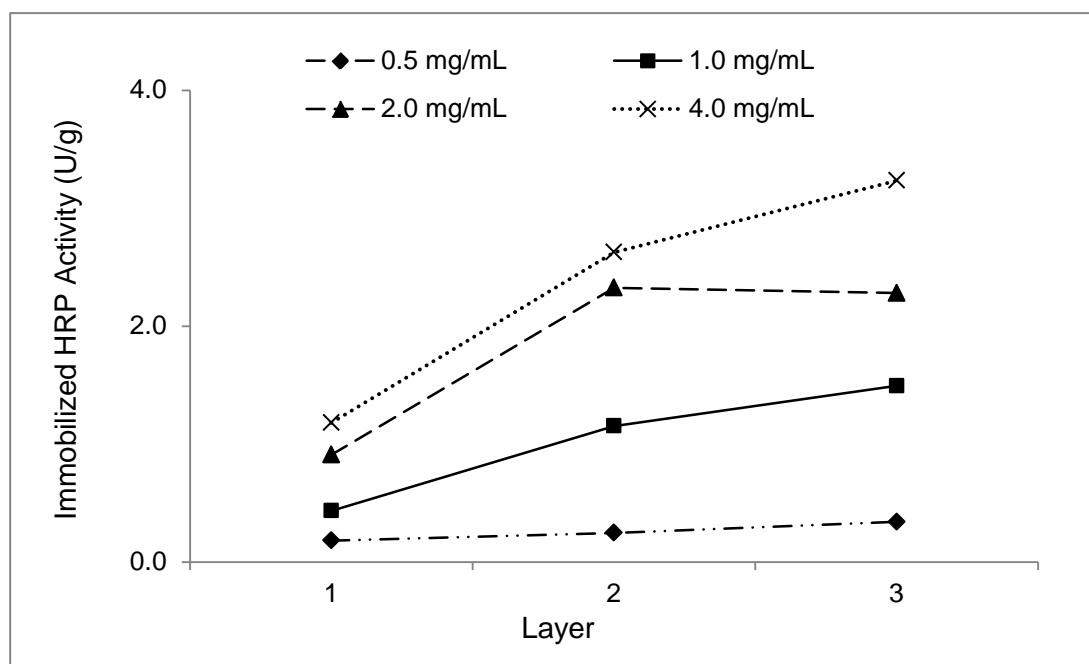

Figure 6. Activity of horseradish peroxidase (HRP) immobilized on sand via layer-by-layer assembly of negatively charged poly (sodium 4-styrenesulfonate) and HRP of various concentration in pH 6.0 buffer solution that rendered HRP positively charged.
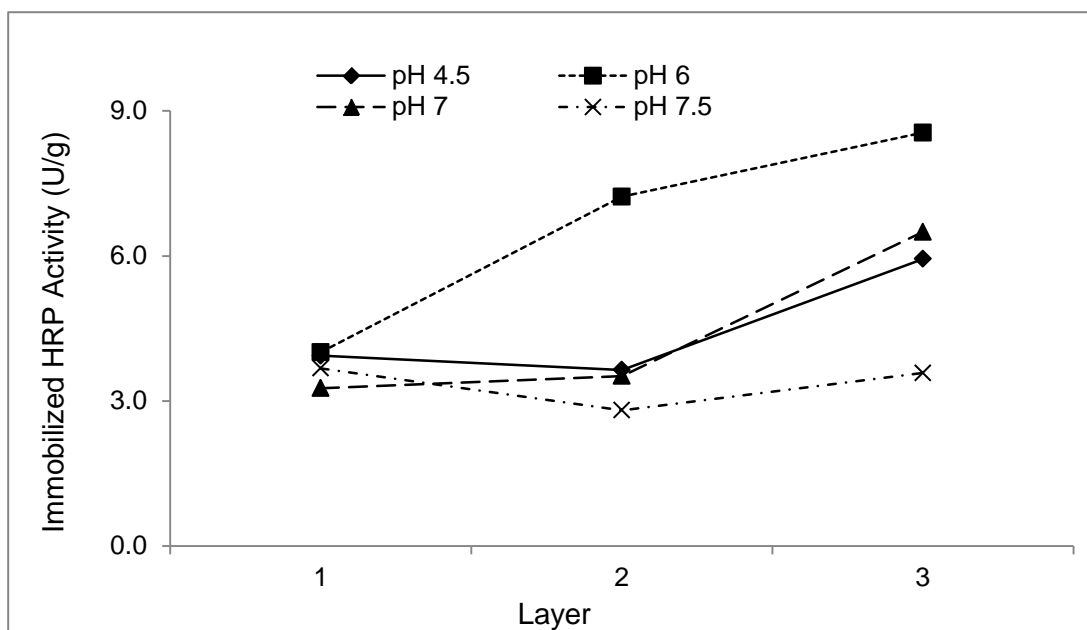
Figure 7. Activity of horseradish peroxidase (HRP) immobilized on sand via layer-by-layer assembly of negatively charged poly (sodium 4-styrenesulfonate) and 2.0 mg/mL HRP in buffer solutions of varying pH that rendered HRP positively charged.
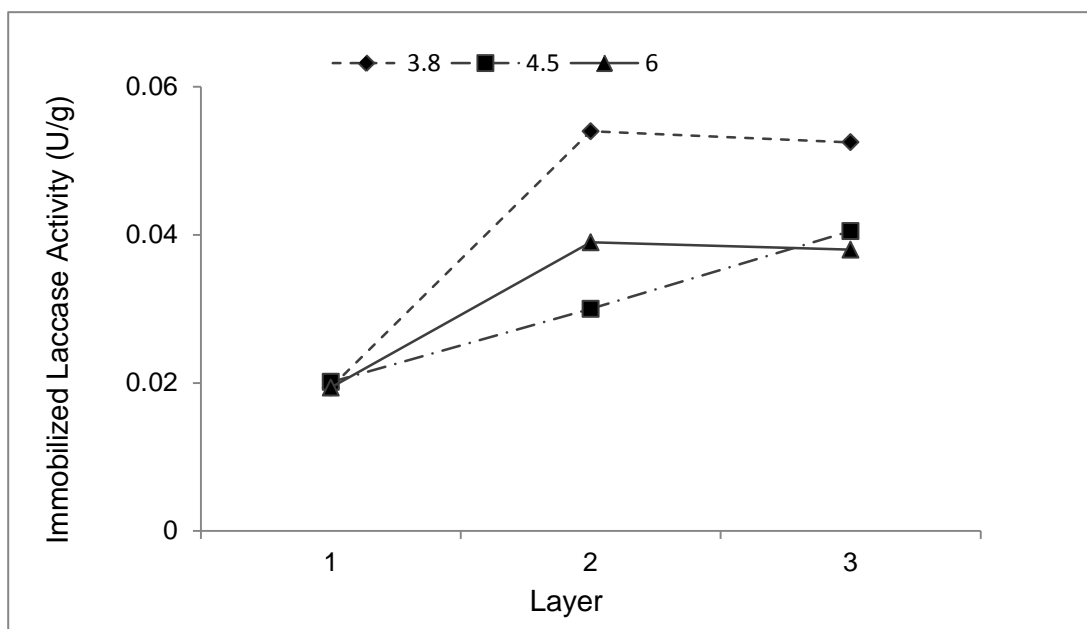

Figure 8. Activity of horseradish peroxidase (HRP) immobilized on sand via layer-by-layer assembly of negatively charged poly(sodium 4-styrenesulfonate) and 10 mg/mL laccase in buffer solutions of varying pH that rendered the laccase positively charged.

<u>Covalent bonding approach</u>

The impact of immobilization temperature and pH on laccase immobilization on sand via covalent bonding was shown in Figure 9. The highest immobilization was achieved when the immobilization was carried out at pH 3.8, which is the optimum pH for free laccase. The optimum temperature for laccase immobilization was 25^oC, with higher (40^oC) or lower (4^oC) temperature leading to decreased laccase immobilization. We also tested the reusability of the immobilized laccase prepared via covalent bounding (Figure 10). Nearly 80% of the activity remained after 6 reaction cycles, indicating the stability of the sand-immobilized enzyme by covalent bonding.
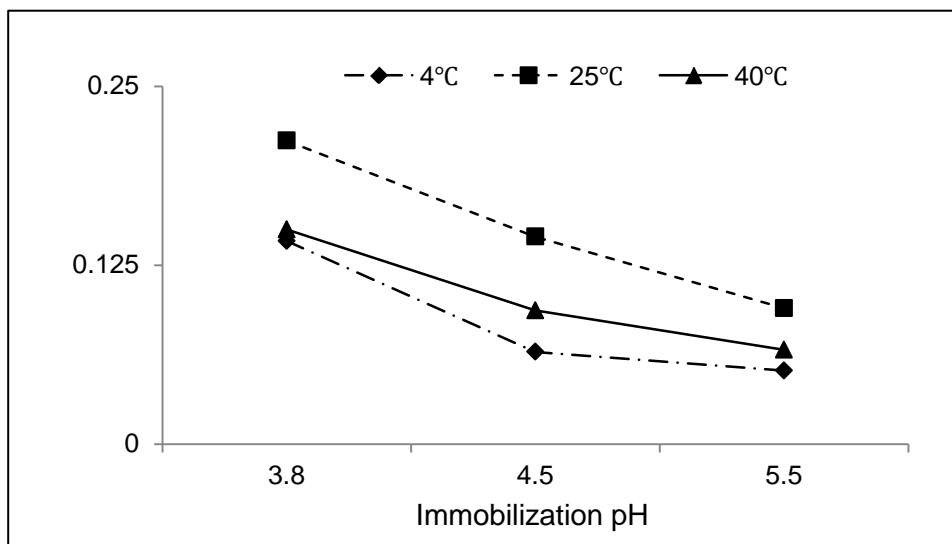

Figure 9. Activity of laccase immobilized on sand via covalent bonding under different pH and temperatures using a 10 mg/mL laccase solution.
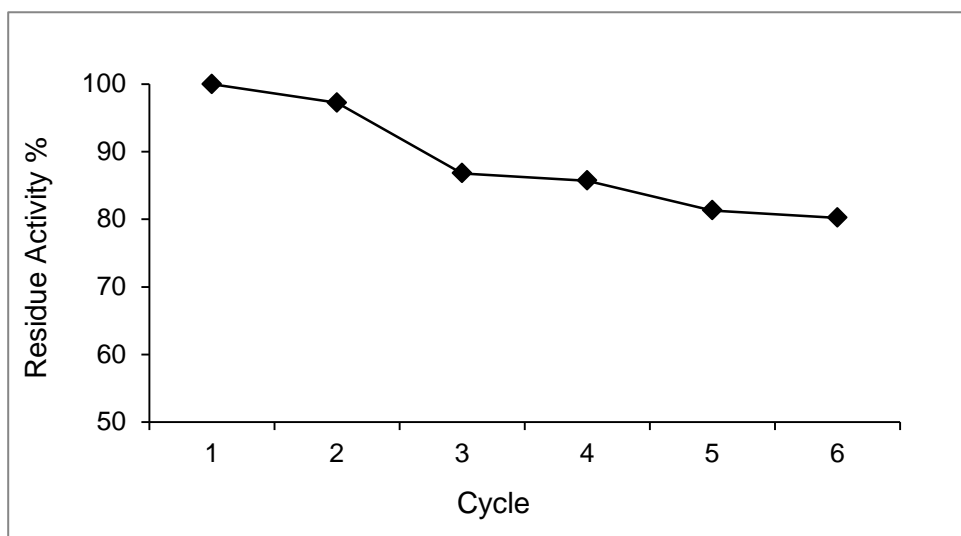

Figure 10. Residual activity of immobilized laccase after multiple reaction cycles with 2, 6-dimethoxyphenol as the substrate.

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Figure 11 depicts immobilized laccase activities on a sandy loam soil and the clay kaolinite under different immobilization pH. The immobilized laccase activities onto the sandy loam soil and the clay were significantly higher than on sand. The immobilized activities at optimum immobilization condition were 3.98 unit/g on the soil and 7.91 unit/g on the clay. The effective laccase immobilization on soil may be attributable to its clay contents. The immobilized laccase activity was greater when it was immobilized at pH 3.8 than pH 7.0. Apparently, the optimum immobilization pH for laccase is close to the optimum pH of free laccase. The stability of laccase immobilized on the soil and the clay were also investigated (Figure 12). Over 21 days, the laccase immobilized on soil and clay did not lose much activity. Base on the above data, covalent bonding seems to be an efficient and reliable approach to immobilize the laccase on soil and clay.
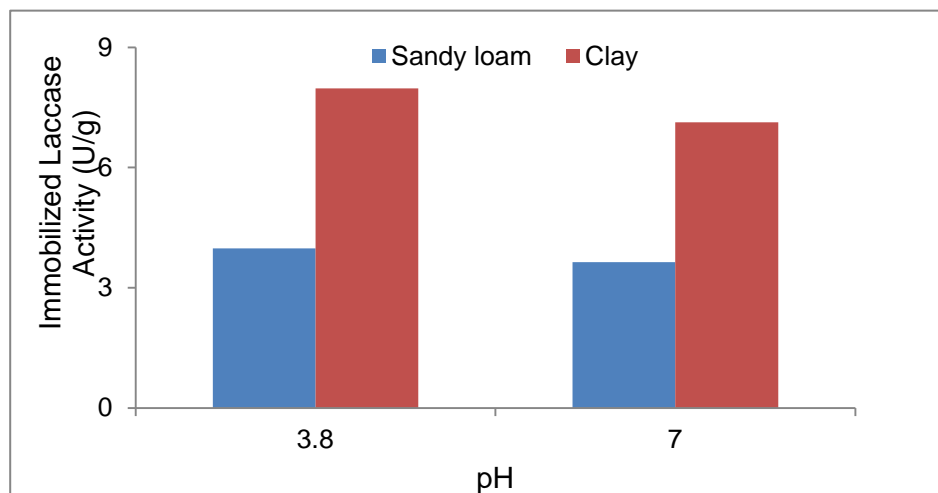

Figure 11. Activity of laccase immobilized on a sandy loam soil and a clay through covalent bonding under pH 3.8 and 7.0.
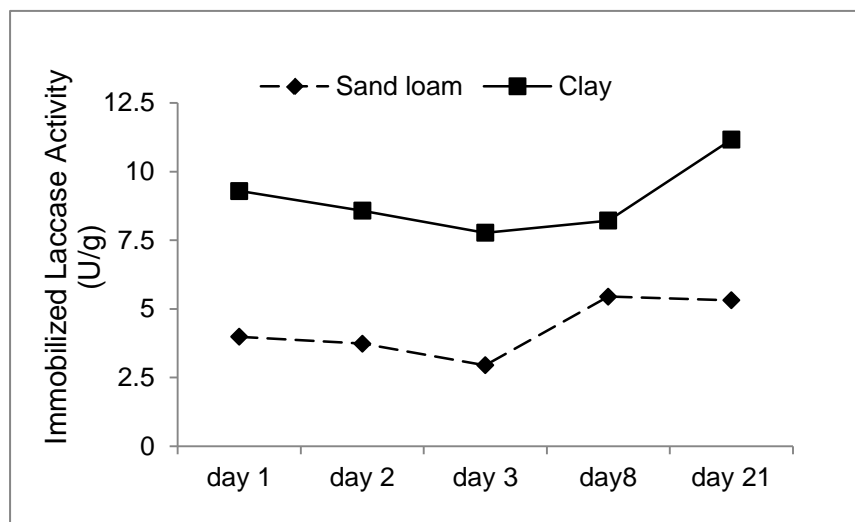

Figure 12. The change of the activity of immobilized laccase on a sandy loam soil and a clay over 21 days of storage at 4°C.

<u>Adsorption</u>

The sorption isotherm of laccase on activated carbon is shown in Figure 13. When the laccase activity in aqueous phase was 0.35 U/mL, the activity adsorbed on GAC achieved 230 U/g. The immobilization efficiency is very high, more than the other immobilization methods and carriers that we have tested. Besides, adsorption approach is relatively simple and inexpensive, therefore having great potential in remediation application.
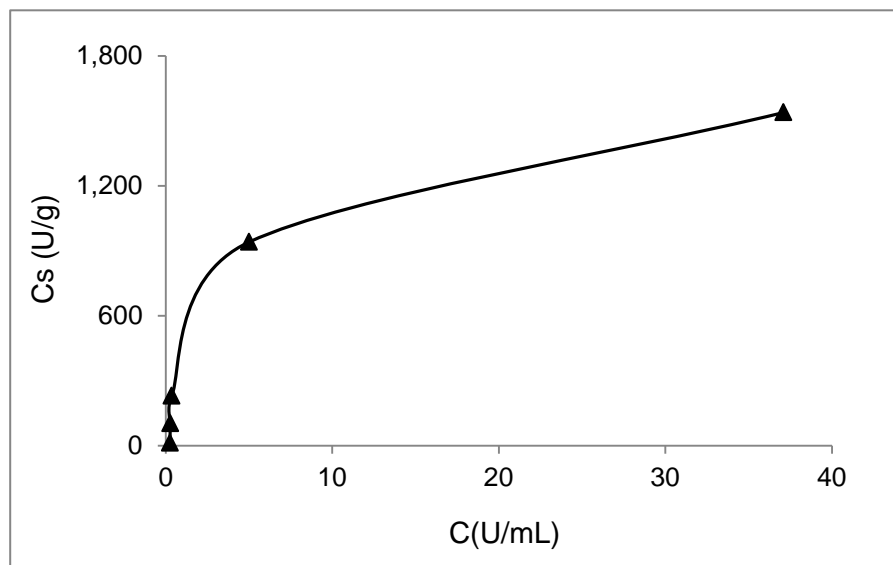

Figure 13. Sorption isotherm of laccase on granular activated carbon at 25°C.

4.3 Conclusion and future research needs

Sand did not seem to be a good support medium for immobilizing humification enzymes, probably because of its limited specific surface area. On the contrary, soil and clay seemed to be good support media. The good immobilization on soil was probably attributable to its clay contents. Adsorption led to effective immobilization of laccase on GAC, making a great option for potential remediation applications. Further study is needed to investigate laccase adsorption on other materials of application potential such as clay, diatom earth, and different types of soils, as well as laccase immobilization by covalent bonding on GAC, different clays and other materials, and to elucidate factors controlling the immobilization efficiency.

5. Column study

We originally proposed a double-layer permeable reactive barrier (DL-PRB) system, shown in Figure 1, for inducing in-situ ECHOR reactions. The DL-PRB comprises an oxidant-releasing material layer followed by a layer of quartz sands immobilized with humification enzymes. Our idea had however evolved based on the batch study and the enzyme immobilization study results discussed above. First, sand is not a good candidate for enzyme immobilization to be used in the reactive barrier. Instead, GAC, clay, or soil, individually or combined, may be good materials for immobilizing enzymes and used in the reactive barrier. Sand may also be mixed in the barrier to adjust flow condition. Second, the oxygen releasing

material, such as calcium peroxide, does not have to be a separate layer prior to the reactive layer, but can be directly mixed in the reactive barrier. The evolved conceptual model is schematically represented in Figure 14. We have conducted a laboratory column study to examine POFA breakthrough on a column packed with GAC immobilized with laccase through adsorption. In order to design this column study, we have also investigated adsorption isotherms of PFOA and a few mediators on GAC.
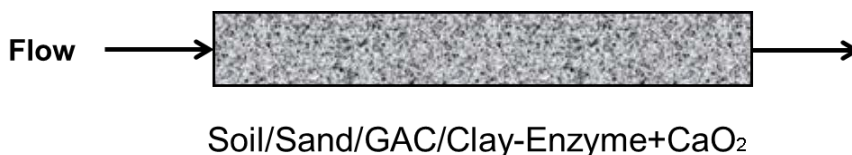

Soil/Sand/GAC/Clay-Enzyme+CaO$_2$

Figure 14. Conceptual model of an ECHOR barrier

5.1 Experiments

5.1.1 Sorption isotherms

Batch sorption experiments were carried out at 25°C by mixing granular activated carbon (GAC) with a chemical solution under consistent shaking at 225 rpm. We have tested the sorption isotherms of four mediators, including 4-methoxyphenol, guaiacol, catechol, and HBT. In these experiments, 10 mg of GAC was mixed with 20 mL of the mediator solution at concentrations ranging from 0.25 mM to 25 mM. After 48 hours of mixing, which was adequate to achieve equilibrium based on preliminary study, 2 mL of the sample was taken and then centrifuged. The supernatant was then transferred to HPLC vial for analysis. The sorption isotherm experiment for PFOA was conducted in 250 mL polyethylene bottles that contained 10 mg GAC and 100 mL of HPLC water spiked with PFOA at concentrations ranging from 1 mg/L to 80 mg/L. The mixture was continuously agitated on an incubator shaker for 7 days. At pre-selected time intervals, samples were taken from the mixture and set still for 1 hour to allow phase separation and the supernatants were sampled for HPLC analysis.

5.1.2 Column study

The setup of the flow-through column experiment is schematically represented in Figure 15. First, laboratory-scale columns packed with 0.1 g GAC and 2 g quartz sand were prepared for the flow-through column experiment. The 2 mL micro-column fitted with a porous polyethylene frit on one end was first wetted with HPLC water and then filled with 0.1 g GAC that was prior saturated with HPLC water. The column was periodically tapped and the water level in the column was maintained above the solid fillings through the packing process to ensure packing quality. On top of this GAC layer, 2 g wetted sand was filled using the same approach, and then another frit was fitted on top of the sand layer to secure the packed beds. After packing, the column was flushed with HPLC water at 1 mL/min for 10 hours before use. In order to preload laccase to the packed column, 42 mL of a 1.0 unit/mL laccase solution was passed through the column using a syringe pump and laccase activity in the effluent was measured at selected time points. For a PFOA flow-through experiment, a mixture solution containing 0.067 mg/L PFOA, 0.15 mM of HBT and 1 unit/mL laccase was continuously passed through the column at a flow rate of 2 mL/min using a Waters HPLC pump. HBT was used as the co-
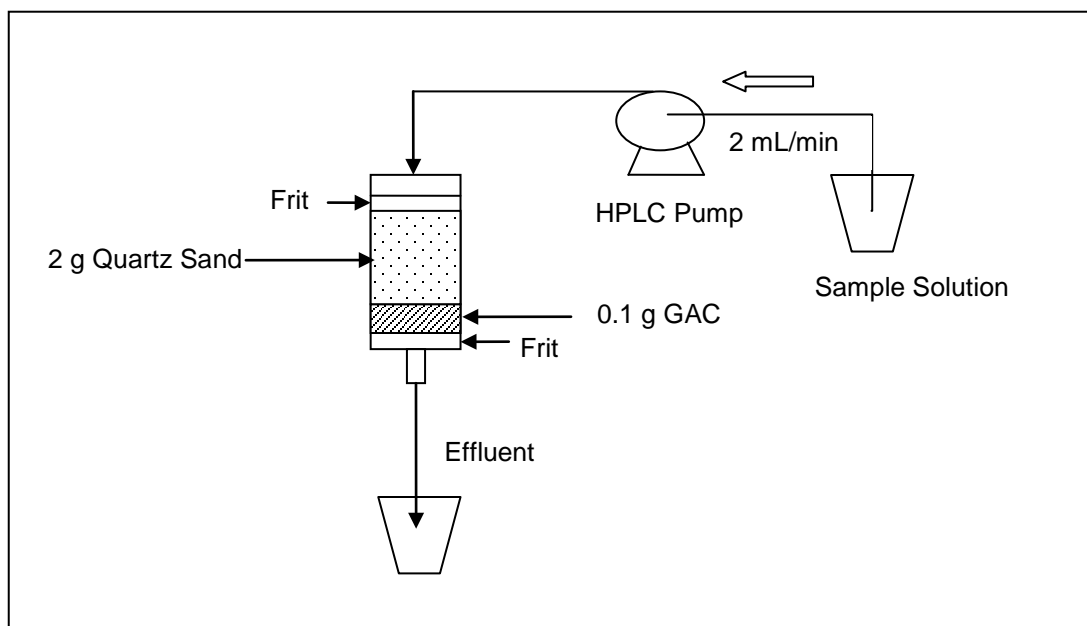
Figure 15. The setup of a GAC micro-column experiment

substrate to mediate ECHOR, and the concentrations were selected based on isotherm and batch studies. The effluent was collected at pre-selected time intervals in conical flask and acidified with 1M HCl solution to stop ECHOR. A blank control column was also prepared in which laccase was not pre-loaded, and the solution used in flow-through experiment contained only 0.067 mg/L PFOA without laccase or HBT. A total of 15 L solution was passed through the column and the procedure lasted about 6 days. Selective effluent samples were concentrated by passing through Waters Oasis HLB cartridges and eluted with methanol, and the eluent was collected and saved for LC-MS/MS analysis to quantify PFOA and HBT.

5.2 Results and discussion

The sorption isotherms of 4-methoxyphenol, guaiacol, catechol, and HBT on GAC are displayed in Figure 16, and all exhibited favorable strong sorption. To facilitate comparison, the solid phase concentrations of the chemicals when their aqueous phase concentration are near 4 mM are 2500, 1823,1638 and 1085 mmole/Kg for catechol, guauacol, 4-methoxyphenol and HBT, respectively. The sorption isotherm of PFOA on GAC is shown in Figure 17a and its data fitting by Freundlich equation is shown in Figure 17b. The sorption of PFOA on GAC is much stronger than the other four chemicals. When the aqueous concentration of PFOA is near 1mg/L, the solid phase concentration is about 100,000 mg/Kg. The sorption isotherm of laccase on GAC is shown in Figure 13. Based on these sorption isotherm data, we have selected the concentration levels of the PFOA (0.06 mg/L), the mediator HBT (0.15 mM) and laccase (1 unit/L) for the feed solution in the flow-through column experiment. These correspond to equilibrium solid phase concentrations of 9.37 mg/g, 196 mmole/Kg, and 477 unit/g, for PFOA, HBT and laccase, respectively.
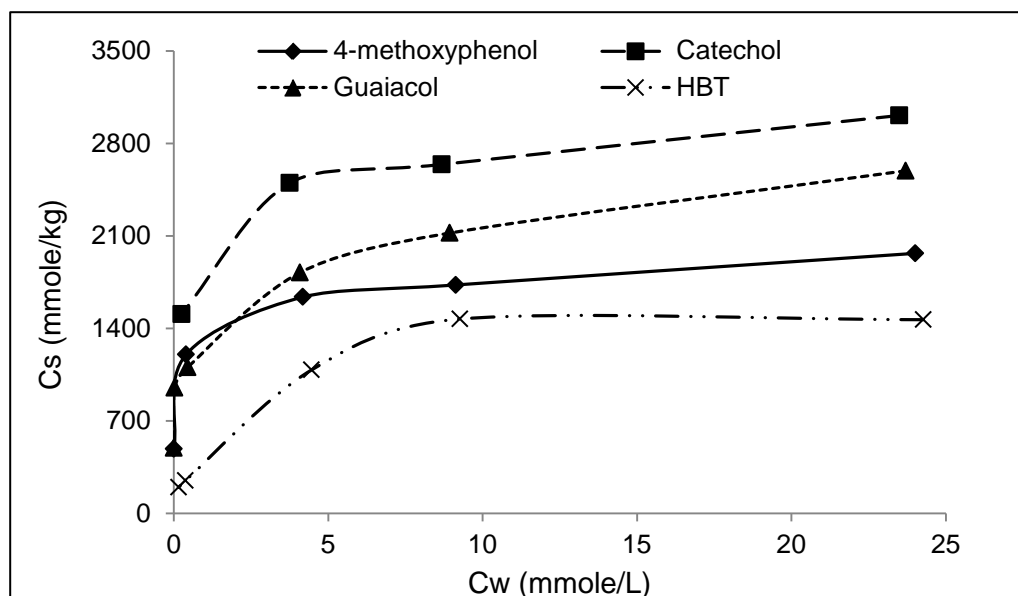
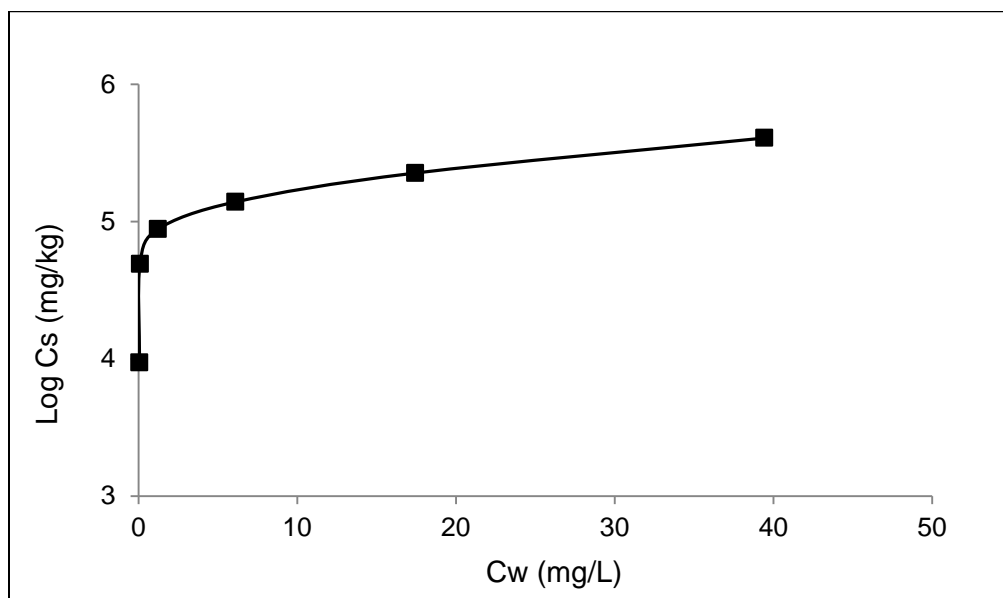Figure 16. Sorption isotherms of 4-methoxyphenol, catechol, guaiacol, and HBT on GAC at 25°C.

Figure 17. Sorption isotherm of PFOA on GAC at 25°C

The laccase activity in the effluent over time during the laccase-preloading procedure by passing a 1.0 unit/L laccase solution through the GAC-packed column is shown in Figure 18. A typical breakthrough curve was obtained and the breakthrough occurred when about 20 mL solution was passed, corresponding to that about 20 units laccase were retained on the 0.1 g GAC packed in the column, assuming the sands does not sorb laccase. The well-shaped laccase breakthrough curve also indicates the good quality of the column packing.
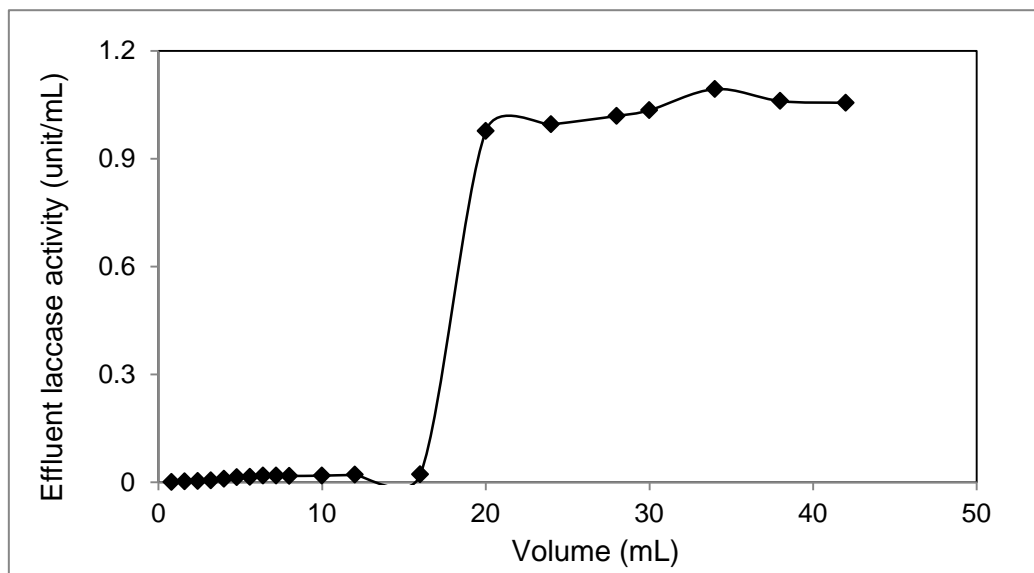
Figure 18. Breakthrough of laccase (1.0 unit/mL) on a GAC (0.1g) packed micro-
column.

A flow-through column experiment was conducted, as described in the Experiments, to compare a reaction system in which laccase was pre-loaded to the column and the feed solution contained PFOA, HBT and laccase and a blank control system in which laccase was not pre-loaded and the feed solution only contained PFOA. As shown in Figure 19, the breakthrough on the reaction column seemed to occur slightly earlier than the blank column, but clearly has the PFOA concentration in the effluent reduced. The enzyme and mediator present in the feed solution for the reaction column may have reduced PFOA sorption on GAC that may have contributed to the quicker PFOA breakthrough on the reaction column. The maximum PFOA concentration in the effluent from the blank column was 0.0664 mg/L while that from the reaction column was 0.0576 mg/L. A calculation based on the breakthrough curve shown in Figure 19 yields the solid phase concentration on the blank and reaction columns were 3.37 and 2.52 mg/g, respectively, representing nearly 25% reduction on the reaction column than the blank column. Note that the column experiment lasted only about 6 days and more reduction may be achieved by longer contact time or by optimizing the conditions to facilitate ECOHR. The breakthrough data in Figure 19 strongly suggest the promising potential of using ECHOR in a PRB setup to remove PFOA in groundwater. More systematic work is needed to identify optimal conditions, such as column size, flow rate, laccase activity, and mediator concentration to facilitate PFOA transformation.
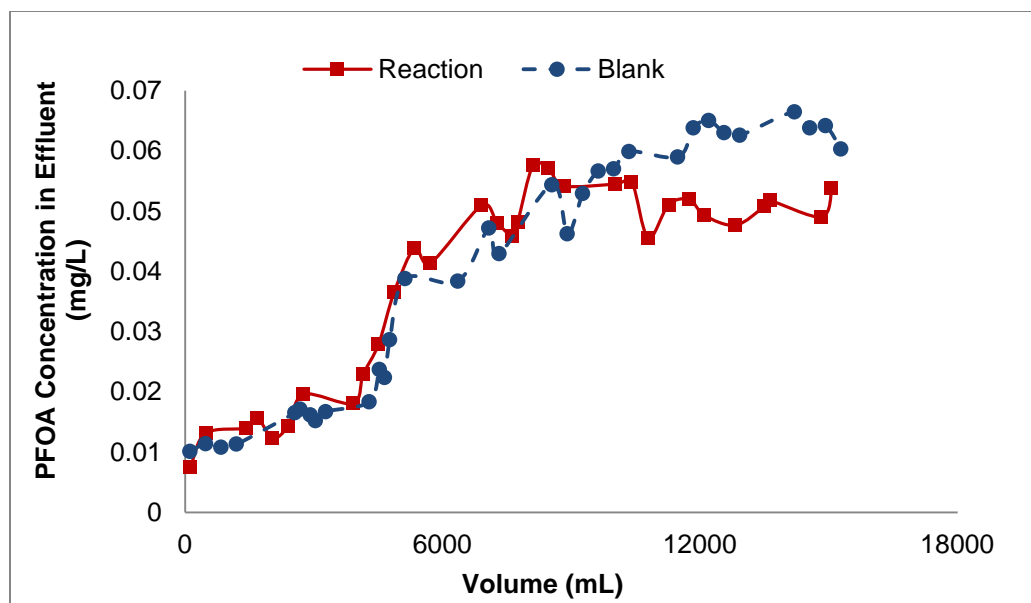
Figure 19. Breakthrough of PFOA on a GAC packed micro-column with ECHOR reaction induced and a blank GAC column. Both columns were packed with 0.1 g GAC, and the reaction column was preloaded with laccase by passing through a 1 unit/mL laccase solution at 0.1 mL/min. The feed solution for the reaction column contained 0.067 mg/L PFOA, 0.15 mM of HBT and 1 unit/mL laccase, while that for the blank column contained only 0.067 mg/L PFOA. The feed solution was operated at 2 mL/min.

5.3 Conclusion and future research needs

PFOA, the mediators and laccase all adsorb strongly on GAC, making is a promising candidate material to be used in PRB to induce ECHOR for PFC remediation. ECHOR may be enhanced on GAC surface through micro-reactor effects because all factors are concentrated through sorption, which should be verified in future study using batch reactor systems. The data from column study strongly suggest the promising potential of using ECHOR in PRB for PFCs remediation in soil/groundwater. More work is needed to identify optimal conditions to facilitate PFCs transformation in PRB. Flow-through column experiments should also be conducted in the future with other support media, e.g. soil, clay, sand, in addition to GAC, individually and in mixture under different conditions with modeling attempts to provide guidance for system design and optimization.

6. Publication

U.S. provisional patent (Serial No. 61/650,075) Enzyme composition and methods to transform perfluoroalkyl compounds in soil, Qingguo Huang, UGARF.

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